Molecular Tools for the Yeast *Papiliotrema terrestris* LS28 and Identification of Yap1 as a Transcription Factor Involved in Biocontrol Activity

Raffaello Castoria,a Cecilia Miccoli,a Giuseppe Barone,a Davide Palmieri,a Filippo De Curtis,a Giuseppe Lima,a Joseph Heitman,b Giuseppe Ianiri,a

aDepartment of Agricultural, Environmental and Food Sciences, University of Molise, Campobasso, Italy
bDepartment of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina, USA

**ABSTRACT** Fungal attacks on stored fruit and vegetables are responsible for losses of products. There is an active research field to develop alternative strategies for post-harvest disease management, and the use of biocontrol agents represents a promising approach. Understanding the molecular bases of the biocontrol activity of these agents is crucial to potentiate their effectiveness. The yeast *Papiliotrema terrestris* is a biocontrol agent against postharvest pathogens. Phenotypic studies suggest that it exerts its antagonistic activity through competition for nutrients and space, which relies on its resistance to oxidative and other cellular stresses. In this study, we developed tools for genetic manipulation in *P. terrestris* to perform targeted gene replacement and functional complementation of the transcription factors Yap1 and Rim101. In vitro phenotypic analyses revealed a conserved role of Yap1 and Rim101 in broad resistance to oxidative stress and alkaline pH sensing, respectively. In vivo analyses revealed that *P. terrestris yap1Δ* and *rim101Δ* mutants display decreased ability to colonize wounded fruit compared to that of the parental wild-type (WT) strain; the *yap1Δ* mutant also displays reduced biocontrol activity against the postharvest pathogens *Penicillium expansum* and *Monilinia fructigena*, indicating an important role for resistance to oxidative stress in timely wound colonization and biocontrol activity of *P. terrestris*. In conclusion, the availability of molecular tools developed in the present study provides a foundation to elucidate the genetic mechanisms underlying biocontrol activity of *P. terrestris*, with the goal of enhancing this activity for the practical use of *P. terrestris* in pest management programs based on biological and integrated control.

**IMPORTANCE** The use of fungicides represents the most effective and widely used strategy for controlling postharvest diseases. However, their extensive use has raised several concerns, such as the emergence of plant pathogens’ resistance as well as the health risks associated with the persistence of chemical residues in fruit, in vegetables, and in the environment. These factors have brought attention to alternative methods for controlling postharvest diseases, such as the utilization of biocontrol agents. In the present study, we developed genetic resources to investigate at the molecular level the mechanisms involved in the biocontrol activity of *Papiliotrema terrestris*, a basidiomycete yeast that is an effective biocontrol agent against widespread fungal pathogens, including *Penicillium expansum*, the etiological agent of blue mold disease of pome fruits. A deeper understanding of how postharvest biocontrol agents operate is the basic requirement to promote the utilization of biological (and integrated) control for the reduction of chemical fungicides.

**KEYWORDS** *Papiliotrema terrestris*, *Penicillium expansum*, *Monilinia fructigena*, biocontrol, oxidative stress
To date, plant protection from fungal pathogens is based mainly on the use of synthetic fungicides. Indeed, the fungicide list approved in Europe includes 169 active ingredients, of which only 10 are considered low-risk type (EPPO 2020). However, the increasing public concerns about their toxic effects on human health and on the environment, along with the progressive reduction of available pesticides and the emergence of resistance in plant-pathogenic fungi, are driving the development of safer technologies for disease management (1, 2). Among them, biological control is a promising technology.

Biological control is defined as the mitigation of pests and diseases through the use of natural antagonists named biocontrol agents (BCAs) (3). Many microorganisms have been reported as potential BCAs for their ability to decrease the development of pathogens. Nevertheless, there is still limited knowledge on the mechanisms that underlie the biocontrol activity of these beneficial microbes, limiting their use in practical pest management programs. As a consequence, chemical fungicides are still the main method of plant pathogen control. Understanding the molecular bases of biocontrol through genomic and transcriptomic approaches, combined with the availability of tools for genetic manipulation, is pivotal to fully exploit the potential of BCAs and to open new avenues for effective biocontrol strategies (4–6).

*Papiliotrema terrestris* is a basidiomycetous yeast belonging to the Tremellaceae in the subphylum Agaricomycotina. The genus *Papiliotrema* was first proposed in 2002 to accommodate the new species *Papiliotrema bandonii* (7), and it has been recently revised (8). *P. terrestris* was described by Crestani et al. (66) as ubiquitous in the soil (hence the name *terrestris*, which means “of the soil” in Latin). The microorganism of interest in the present work is strain LS28 of *P. terrestris*, previously reported as *Cryptococcus laurentii* and recently reclassified following phylogenetic analyses (9). The biocontrol activity of *P. terrestris* LS28 on stored fruits is mainly preventive and is based on competition for nutrients and space, which relies on the rapid colonization of fruit wounds (wound competence), the main penetration sites of pathogens within host tissues (10–15). This is considered the main mode of action of the majority of yeast BCAs, and it relies on the natural nutritional requirements of these beneficial microbes (16, 17). Further studies revealed that tolerance/resistance to reactive oxygen species (ROS: superoxide anion \([O_2^-]\) and hydrogen peroxide \([H_2O_2]\) generated by plant tissues as a consequence of wounding or by certain pathogens (i.e., *Botrytis cinerea*) as virulence factors (18), is critical for wound competence (12, 19, 20).

Although competition for space and nutrients is the main mode of action of *P. terrestris* LS28 and other yeast BCAs, other mechanisms that might be involved in biocontrol were also reported. The production of antibiotic compounds, an undesirable mode of action of many bacterial BCAs, was not detected in *P. terrestris* LS28 (10), but other mechanisms could also play a role in the biocontrol activity, such as the production of enzymes (\(\beta\)-1,3-glucanases and chitinases) depolymerizing fungal cell walls (10, 21), the induction of resistance in the host tissues (22, 23), and the expression of genes encoding carbohydrate-active enzymes (24).

*P. terrestris* LS28 is also known for its compatibility with synthetic fungicides, displaying elevated resistance to several active compounds. Based on these phenotypic features, this BCA displayed elevated antagonistic activity when applied in combination with full or low dose of synthetic fungicides, clearly demonstrating its potential for practical use in integrated disease management programs (11, 13, 14, 25, 26).

In the present study, we developed new tools to study the molecular bases underlying biocontrol activity of the BCA *P. terrestris* LS28. In particular, we (i) developed specific dominant drug resistance genetic markers that encode resistance to hygromycin B and neomycin G418, (ii) used *Agrobacterium*-mediated transformation, biolistics, and electroporation to deliver the *HYG* and *NEO* markers, (iii) performed a reverse genetics approach based on electroporation and biolistics to generate targeted deletion mutants for the *P. terrestris* LS28 transcription factors Yap1 and Rim101, and (iv) characterized the *yap1Δ* and *rim101Δ*
mutant phenotypes in vitro, as well as in vivo during interaction with the fungal pathogens Penicillium expansum and Monilinia fructigena on wounded apples.

RESULTS
Development of transformation systems for P. terrestris LS28. For the generation of molecular tools for P. terrestris LS28, it was critical to test its sensitivity to the most common antifungal drugs used for selection in genetics experiments. The concentrations of hygromycin B (HYG), neomycin G418 (NEO), and nourseothricin (NAT) that completely inhibit the growth of P. terrestris LS28 on solid YPD medium (yeast extract at 10 g/liter, peptone at 20 g/liter, dextrose at 20 g/liter, and agar at 20 g/liter for solid medium) were determined using a range of antifungals from 100 to 450 μg/ml. Concentrations of 200 μg/ml of HYG, 300 μg/ml of NAT, and 450 μg/ml of NEO, coupled with incubation at 30°C, were sufficient to completely inhibit the growth of WT P. terrestris LS28.

Before developing markers specific for P. terrestris LS28, the HYG, NEO, and NAT cassettes commonly applied for genetic manipulations of the closely related basidiomycete Cryptococcus neoformans were tested (27, 28). Agrobacterium-mediated transformation (AMT), biolistics, and electroporation of P. terrestris LS28 were carried out as reported for C. neoformans (27, 29, 30). C. neoformans strain H99 served as the positive control, whereas negative controls were water for electroporation and biolistics, as well as the untransformed Agrobacterium tumefaciens EHA105 for AMT. Transformants were obtained only for C. neoformans, and no colonies were obtained for P. terrestris LS28 (see Fig. S1 in the supplemental material).

Therefore, specific gene markers for P. terrestris LS28 were generated through cloning into the binary vector pGI3 the NAT, NEO, and HYG coding sequences under the control of the promoter and terminator of the P. terrestris LS28 histone H3 gene. Binary vectors were transformed in A. tumefaciens for AMT, while for biolistics and electroporation, the expression cassettes were amplified by PCR and transformed into P. terrestris LS28. Several parameters were tested as described in detail in Materials and Methods.

Transformation with the NAT marker gave unreliable results, regardless of the transformation method used. P. terrestris NAT-resistant colonies were isolated also on control plates (i.e., water controls for electroporation and biolistics, as well as untransformed A. tumefaciens for AMT), and they displayed cross-resistance to the antifungal NEO but not to HYG (Fig. 1A). PCR analysis could not confirm successful transformation in any of the NAT-resistant colonies. For these reasons, the NAT marker could not be used as a reliable tool for mutagenesis in P. terrestris and was excluded from further experiments.

In the case of the NEO marker, real P. terrestris transformants could be obtained with all three transformation approaches (data not shown). However, because some spontaneous resistance could be observed (see, for example, Fig. 1A), the NEO cassette was used as secondary marker for generating complemented strains as described below. Transformation with the HYG marker achieved the best results because spontaneous resistance was never observed. For AMT, the highest number of transformants (~500 per transformation plates) was obtained with the use of nitrocellulose membranes that facilitate cell-to-cell contact, combined with 6 days of coincubation on induction medium (IM). No HYG+ transformants of P. terrestris LS28 were obtained after 3 days of coincubation, which is the standard used for C. neoformans, likely reflecting the slower growth of P. terrestris LS28 than C. neoformans on induction medium. For electroporation, the conditions that gave the best results were the same as those recently reported for C. neoformans (0.45 kV, 125 μF, and 600 Ω) (30), with the only variation being the recovery time of 4 h at 30°C; transformation efficiency was 220 ± 12 transformants per μg of DNA. For biolistics, the best results were obtained with rupture discs of 1350 lb/in² and recovery at 30°C for 4 h; transformation efficiency was 30 ± 10 transformants per μg of DNA. Representative images of the transformation plates of P. terrestris LS28 with the HYG marker are reported in Fig. 1B. The stability of the exogenous HYG marker within the genome was confirmed at the phenotypic level.
by assessing the ability of ~100 P. terrestris putative transformants to display HYG resistance after five consecutive passages in drug-free medium. Corroborating previous studies (27), transformants obtained by AMT displayed the highest stability (>98%), followed by those obtained with biolistic (94%) and electroporation (88%).

For molecular confirmation of P. terrestris LS28 transformants, 10 randomly selected HYG-resistant colonies obtained with the three transformation systems were subjected to PCR analysis to assess the presence of the exogenous HYG marker before and after the stability test. For AMT, 10 out of 10 transformants showed the expected amplicon both pre- and post-stability test; for electroporation, 2 transformants showed a different amplification pattern after the stability test, likely suggesting spontaneous acquisition of HYG resistance in these strains; for biolistics, 1 transformant did not show the expected amplicon either pre- or post-stability test, also suggesting spontaneous and stable acquisition of HYG resistance in this strain (Fig. 1C).

**Generation of targeted yap1Δ and rim101Δ mutants of P. terrestris LS28.** Given the known role of resistance to oxidative and other stresses for successful antagonistic activity of the BCAs (12, 20, 31), targeted mutagenesis approaches in P. terrestris LS28 were tested for two known stress-responsive transcription factors, encoded by YAP1 and RIM101. Transformations were performed using both biolistics and electroporation in combination with the HYG marker, and the rationale is that these techniques are routinely employed for targeted mutagenesis in the closely related pathogenic yeast C. neoformans (27, 32). In the model yeast Saccharomyces cerevisiae, Yap1 is a
nonessential basic leucine zipper (bZIP) transcription factor required for stress resistance, in particular for oxidative stress tolerance (33). The P. terrestris YAP1 gene (GenBank accession MW298104) was identified in the unpublished genome assembly through BLAST analysis; we noticed that the predicted P. terrestris YAP1 gene was larger than any other ortholog deposited in GenBank, and we therefore manually inspected the accurateness of the automatic gene model. Using unpublished transcriptome sequencing (RNA-seq) evidence, we found that the predicted YAP1 gene had an additional 3' region of ~1,500 bp, including the first exon of the adjacent gene that was accordingly removed, and the YAP1 gene model and the Yap1 protein prediction were corrected (Fig. S2). The revised P. terrestris Yap1 was then subjected to domain analysis, which revealed the presence of classical Yap1 domains: an N-terminal bZIP domain that contains a region mediating sequence-specific DNA binding, followed by a leucine zipper required for dimerization, including also a nuclear localization signal (NLS; predicted based on comparison with C. neoformans as RRKEQNRA [Fig. S3]), and a cCRD cysteine-rich domain at the C terminus (named also Yap1 redox domain) for redox regulation, which contains a nuclear export signal (NES; predicted based on comparison with C. neoformans as LIDDLCDM [Fig. S3]). The same domains are present also in C. neoformans and in Schizosaccharomyces pombe Pap1 (Pap1 is the ortholog of Yap1), while S. cerevisiae Yap1 has an additional cysteine-rich domain at the N terminus (name nCRD). Lastly, different software (InterPro Scan, Conserved Domain Database) failed to predict the cCRD domain in Ustilago maydis, although this was reported by Mendoza-Martínez et al. (34) (Fig. 2A).

**FIG 2** Targeted gene replacement of the YAP1 gene in P. terrestris LS28. (A) Domain analysis of the Yap1 protein in P. terrestris (Pt), C. neoformans (Cn), S. cerevisiae (Sc), S. pombe (Sp), and U. maydis (Um) For further details, see Fig. S3. (B) Schematic representation of the gene deletion strategy by homologous recombination. At the top is a schematic representation of the YAP1 gene of P. terrestris LS28, with (in purple) the primers used to obtain flanking regions for homologous recombination and (in blue) internal primers used for diagnostic PCR shown in panel C. The bottom shows the YAP1 gene replaced with the HYG marker, with (in red) the primers used for diagnostic PCR shown in panel C. (C) Molecular analyses to identify correct yap1ΔHYG mutants in transformants obtained through biolistics and electroporation. The primers used are indicated, and their position is represented in panel B; only transformants showing the expected amplification pattern in all four PCR analyses were considered correct yap1Δ mutants of P. terrestris LS28, and they are indicated with the red symbol “Δ”; the ladder indicates the amplicon size. (D) Phenotypic characterization of two independent yap1Δ mutants of P. terrestris LS28, one obtained through biolistics and one by electroporation, and a complemented yap1Δ + YAP1 transformant. Stressors included hydrogen peroxide (H₂O₂; 3 mM), tert-butyl hydroperoxide (t-BOOH; 0.5 mM), methyl methanesulfonate (MMS; 0.5 mg/ml), and sodium nitrite (NaNO₂; 0.125 mM); HYG and NEO were used as a control.
For targeted gene replacement of YAP1 in *P. terrestris* LS28, primers were designed to replace a region of 1,845 bp with the HYG marker (Fig. 2B). Twenty-four HYG-resistant transformants, 12 obtained with biolistics and 12 with electroporation, were screened by PCR for homologous recombination events. Analysis of the junctions at the 5′ and 3′ ends revealed the presence of the expected amplicons of ~4.3 kb for each side in several transformants. Furthermore, we exploited the 1,174-bp size difference between the HYG marker (3,019 bp) and the replaced region of the YAP1 gene (1,845 bp) to further confirm the correct event of homologous recombination, resulting in the generation of a 5.3-kb amplicon for the wild type (WT) and a 6.5-kb amplicon in the *yap1Δ HYG* mutants. Lastly, primers designed within the YAP1 gene were also used, resulting in the generation of a ~1-kb amplicon only for transformants that still had a WT copy of the gene. These four different diagnostic PCRs led to the final selection of five unequivocal *P. terrestris* LS28 *yap1Δ* mutants, three obtained with biolistic transformation and two with electroporation (Fig. 2C). One *P. terrestris* LS28 *yap1Δ HYG* mutant was transformed with a YAP1-NEO construct to generate complemented strain *yap1Δ + YAP1*.

Two independent *yap1Δ* mutants, one obtained with biolistics and one obtained with electroporation, and a complemented *yap1Δ + YAP1* strain were tested for their sensitivity to several stressing conditions. The *yap1Δ* mutant exhibited high sensitivity to oxidative stress-inducing agents hydrogen peroxide and tert-butyl hydroperoxide and to the genotoxic stress-inducing agent methyl methanesulfonate (MMS), as well as a slight sensitivity to the nitrosative stress-inducing agent sodium nitrite. As expected, reintroduction of the YAP1 gene restored stress tolerance at the wild-type level, unequivocally indicating that *P. terrestris* YAP1 is involved in oxidative, nitrosative, and genotoxic stress (Fig. 2D).

Regarding the gene RIM101, in fungi it encodes a Cys2His2 zinc finger transcriptional repressor involved in alkaline responsive gene repression as a part of adaptation to alkaline conditions. The *P. terrestris* RIM101 gene (GenBank accession number MW298103) was identified in the unpublished genome assembly through BLAST analysis. Domain analysis of Rim101 in the basidiomycetes *P. terrestris*, *C. neoformans*, and *U. maydis* showed that this protein is highly conserved and is characterized by the presence of a zinc finger C2H2-type domain in proximity of the N-terminal region. *S. cerevisiae* Rim101 contains a zinc finger C2H2 domain in the central region of the protein (Fig. 3A).

For mutagenesis of RIM101 in *P. terrestris* LS28, primers were designed to replace a region of 702 bp with the HYG marker. Primers for gene replacement were designed on the *RIM101* sequence identified following BLAST analyses before the *P. terrestris* annotation was available, and therefore, only the conserved region including the functional zinc finger C2H2 domain was targeted (Fig. 3B). Using the same rationale as for the YAP1 gene, 24 HYG-resistant transformants, 10 obtained with biolistics and 14 with electroporation, were subjected to diagnostic PCRs to identify correct homologous recombination events. Spanning PCR in conjunction with PCR to detect correct recombination at the 5′ and 3′ junctions allowed the identification of five unequivocal *P. terrestris* LS28 *rim101Δ* mutants, three obtained with biolistics transformation and two with electroporation (Fig. 3C). One *rim101Δ* mutant was used as recipient for electroporation of a *RIM101-NEO* complemented construct. Two independent *rim101Δ* mutants, one obtained with biolistics and one obtained with electroporation, and a *rim101Δ HYG RIM101-NEO* complemented strain were subjected to *in vitro* phenotypic characterization. Both *rim101Δ* mutants displayed sensitivity to alkaline stress at pH 9 and to the cell wall-damaging agent Congo red (CR); reintroduction of the RIM101 gene restored stress tolerance at the wild-type level (Fig. 3D).

In *vitro* growth, wound competence, and biocontrol activity of *yap1Δ* and *rim101Δ* mutants of *P. terrestris* LS28 against *P. expansum* and *M. fructigena*. The growth of the *P. terrestris* WT, *yap1Δ* and *rim101Δ* mutants, and complemented strains was tested in *vitro* in YPD rich medium (Fig. 4A), yeast nitrogen base (YNB) minimal medium (Fig. 4B), and apple mimicking medium (Fig. 4C). All the strains tested displayed a more robust growth in YPD than in YNB, while in apple mimicking they displayed the slowest growth kinetics. However, regardless of the media employed, all strains displayed
similar growth kinetics, indicating that the genetic manipulations carried out did not cause any fitness defect. Next, the ability of the *P. terrestris* strains to colonize artificial apple wounds was evaluated (Fig. 4D). The cellular concentration of all *P. terrestris* strains tested rapidly decreased after 30 min in fresh apple wounds, followed by a slight increasing trend after 3 h of incubation. At 6 h and 24 h of incubation, there were no significant differences between the *P. terrestris* strains tested. Conversely, after 48 h of incubation, both yap1Δ and rim101Δ mutants displayed a significantly lower ability to colonize apple wounds, both with an average of $3.1 \times 10^5$ CFU/wound compared to the WT ($5.3 \times 10^5$ CFU/wound) and the yap1Δ + YAP1 ($5.7 \times 10^5$ CFU/wound) and rim101Δ + RIM101 ($4.2 \times 10^5$ CFU/wound) complemented strains (Fig. 4D). Raw output of the statistical analysis performed is reported in Data Set S1.

Next, the biocontrol activity of the *P. terrestris* WT, yap1Δ, rim101Δ, and respective complemented strains was tested against the postharvest pathogens *P. expansum* (Fig. 5A and B) and *M. fructigena* (Fig. 5C). Experiments were carried out using a biocontrol agent (BCA) concentration of $5 \times 10^6$ CFU/ml (i.e., $1.5 \times 10^6$ CFU per wound). Against *P. expansum*, an increase of lesion diameters could be observed only for the yap1Δ compared to the WT, although this did not reach statistical significance ($P=0.1256$) (Data Set S2) (Fig. 5A). Biocontrol activity of the yap1Δ strain against *P. expansum* was significantly lower than those of the WT, yap1Δ + YAP1 complemented strain, and rim101Δ mutant (Fig. 5B). Regarding antagonistic assays against *M. fructigena*, disease severity (i.e., lesion diameters) could not be quantified because lesions were irregular and not circular. Also, we noticed that in some cases the brown rot developed from inside the apple tissue, without apparent decayed areas until an advanced disease stage. For this reason, the brown rot disease progression was stopped at the 10th day of incubation, when all artificial wounds showed clear disease symptoms. As regards the biocontrol assay, interestingly, also in this case the antagonistic activity of the *P. terrestris* yap1Δ mutant against *M. fructigena* was significantly lower than that of the
WT and the yap1Δ + YAP1 complemented strain, with apples that in several cases had 100% infected wounds (86.1 ± 12.8) (Fig. 5C). Raw output of the statistical analyses performed for the biocontrol assays data are reported in Data Sets S3 and S4.

DISCUSSION

Here, we report the genetic manipulation of the basidiomycete yeast P. terrestris LS28, a species of biotechnological interest for its ability to act as a biocontrol agent, i.e., to prevent postharvest diseases of fruit caused by phytopathogenic fungi (10, 11). The development of a system for mutagenesis in P. terrestris LS28 is crucial to carry out gene function studies to elucidate the molecular mechanisms that are involved in biocontrol activity.

Initially, the cassettes for genetic manipulation of the basidiomycete yeast C. neoformans were tested in P. terrestris LS28, but unfortunately, no transformants were obtained (Fig. S1). This was an unexpected finding, since both Papiliotrema and Cryptococcus belong to the Tremellales order and are closely related from a phylogenetic viewpoint (8). Nevertheless, we had previously observed high specificity of the regulatory regions necessary for heterologous gene expression even in basidiomycete strains belonging to the same family, such as Sporobolomyces and Rhodosporidium within the Sporidiobolales (35). Therefore, novel expression cassettes were generated by cloning the NAT, NEO, and HYG markers under the control of P. terrestris LS28 histone H3 promoter and terminator and tested for their effectiveness with the most common transformation techniques employed in fungi, such as Agrobacterium-mediated
transformation (AMT), electroporation, and biolistics. Regardless of the transformation method, the HYG marker was found to be the most effective mutagen in *P. terrestris*, also due to the strong antifungal activity of the hygromycin B that hindered the emergence of drug-resistant spontaneous isolates. The NEO marker was effective, although

**FIG 5** The YAP1 transcription factor is involved in the biocontrol activity of *P. terrestris* LS28 against *P. expansum* and *M. fructigena*. (A) Disease severity is expressed as lesion diameters (cm) of apple wounds inoculated with *P. terrestris* strains and subsequently with *P. expansum*. Each dot represents the average diameters of a biological replicate (4 infected wounds per apple) which were compared to each other using one-way ANOVA with Tukey’s multiple-comparison test. (B and C) Percentage of protection of artificial apple wounds by *P. terrestris* strains against *P. expansum* (B) and *M. fructigena* (C), calculated as number of protected wounds on the total of infected wounds after 7 and 10 days from the beginning of the experiment, respectively. Each dot represents the average percentage of biocontrol activity of one biological replicate (3 apples each having 4 wounds). For each tested strain, the means of each replicate were compared to each other using one-way ANOVA with Tukey’s multiple-comparison test. Symbols indicate significance as follows: *, P < 0.05; **, P < 0.01; and ***, P < 0.001. The controls water and pathogens alone are represented by full black circles.
we used it as a secondary marker due to the appearance of some NEO’ spontaneous isolates (Fig. 1A). The NAT marker was not employed in this study due to the high rate of onset of spontaneous NAT-resistant isolates and the inability to confirm by PCR its integration in the genome of *P. terrestris*. The transformation protocol employed followed those of other basidiomycete yeasts, such as *Sporobolomyces* spp., *Rhodosporidium* spp., *Phaffia rhodozyma*, and *C. neoformans* (27, 29, 30, 35–37). For biolistics and electroporation, we found that the protocols developed for *C. neoformans* were efficient also in *P. terrestris* LS28, with minor modifications such as the longer recovery times due to the slower growth of *P. terrestris* LS28 than *C. neoformans*. Similarly, successful AMT was achieved by using longer *Agrobacterium*-Papililotrema coinoculation time on IM agar medium, with the highest efficiency obtained after 6 days of coincubation. Intriguingly, we found that specific markers developed for *P. terrestris* LS28 were instead functional in *C. neoformans* (data not shown). This finding is similar to what we previously found within the Sporidiobolales (35), suggesting that differences in the mechanisms of gene regulation exist within the basidiomycetes, which might be related to nucleotide sequence specificity and/or codon usage (35, 38).

With the availability of functional genetic markers and efficient transformation methods, we next tested whether the generation of targeted mutants would be suitable in *P. terrestris* LS28. The genes chosen for targeted mutagenesis were *YAP1* and *RIM101*. Yap1 is a basic leucine zipper (bZIP) transcription factor required for oxidative stress tolerance in fungi. The rationale for selecting this gene is that resistance to stresses, in particular resistance to oxidative stress, has been shown to be an important requirement for successful biocontrol activity (12, 20, 31). Rim101 is a Cys₂His₂ zinc finger transcriptional repressor involved in alkaline responsive gene repression, and the rationale behind this choice is that we have unpublished evidence on the role of pH regulation in the biocontrol activity of *P. terrestris* LS28. The *P. terrestris* LS28 *YAP1* and *RIM101* genes were readily deleted using the *HYG* marker through both biolistics and electroporation. In agreement with previous studies on basidiomycete fungi, a higher rate of homologous recombination was achieved with biolistics than with electroporation (27, 39). The ability to generate targeted gene deletion mutants through AMT is currently under evaluation. Our approach using the *NEO* gene as a secondary marker to generate complemented *yap1Δ* + *YAP1* and *rim101Δ* + *RIM101* strains was successful and reduced the risk associated with the emergence of NEO-resistant spontaneous isolates because of the combined effect of NEO and HYG.

We found that the *P. terrestris* LS28 *yap1Δ* mutants exhibited sensitivity to H₂O₂, tert-butyl hydroperoxide (t-BOOH), MMS, and NaN₃, suggesting a central role for the Yap1 transcription factor in resistance to oxidative, genotoxic, and nitrosative stresses (Fig. 2). Domain analysis revealed conserved features in both representative ascomycetes and basidiomycetes, with the exception of *S. cerevisiae*, consistent with a conserved function of Yap1 in fungi (34, 40–42). In agreement, a recent study in the closely related yeast *C. neoformans* revealed that *yap1Δ* mutants are hypersensitive to oxidative stress-inducing agents H₂O₂, t-BOOH, menadione, and diamide and to methylglyoxal, a toxic product of glycolysis. Moreover, transcriptomic analysis revealed that Yap1 plays a pleiotropic role in diverse biological processes, and it regulates the expression of several genes that function in responses to stresses (43). Furthermore, in *S. cerevisiae* Yap1 is activated by oxidative stress, and *yap1Δ* null mutants are hypersensitive to a number of compounds that induce production of reactive oxygen species (ROS) (42).

Regarding *RIM101*, we found conserved features in basidiomycetes and slightly different rearrangement in the model ascomycete *S. cerevisiae* (Fig. 3A). The *P. terrestris* LS28 *rim101Δ* mutant exhibited no growth at alkaline pH (pH9) and showed sensitivity to Congo red, which induces cell wall stress through inhibition of glucans and chitin synthesis and osmotic destabilization (44). These phenotypes are similar to those obtained in *C. neoformans* (45), *S. cerevisiae* (46), and other filamentous fungi, for which the *RIM101* gene is designated *PacC* (47), and are consistent with a conserved role for *RIM101* in alkaline response and cell wall regulation and remodeling.
We further tested the *P. terrestris* LS28 *yap1Δ* and *rim101Δ* mutants for their ability to colonize apple wounds and for their biocontrol activity against the postharvest pathogens *P. expansum* and *M. fructigena*. We found that after 48 h of incubation, both the *yap1Δ* and *rim101Δ* mutants displayed a lower ability to colonize apple wounds than did the WT and the respective complemented strains (Fig. 4D). Interestingly, these mutants did not show any fitness defect in apple mimicking medium (Fig. 4C), which recalls the same conditions as the wound colonization experiment with the exception of the biochemical response of the host to the wounding that is based on oxidative burst. Indeed, our findings corroborate phenotypic studies of Castoria et al., Zhang et al., and others who reported that the ability of biocontrol yeasts to resist to oxidative stress is crucial for rapid and timely colonization of apple wounds and for outcompeting fungal pathogens (12, 20, 48, 49). Accordingly, we found that only the *yap1Δ* mutant displayed lower antagonistic activity against *P. expansum* and *M. fructigena*, further validating wound competence as a critical requirement of a successful biocontrol agent (Fig. 5). Overall, our results indicate that broad resistance to oxidative stress is a critical mechanism of biocontrol activity of *P. terrestris* LS28. In agreement, Sui and colleagues reported that in *Candida oleophila*, mutation of transcription factor Rml1 resulted in increased sensitivity to heat, salt, and oxidative stress, with consequent lower wound colonization rate and biocontrol activity against *B. cinerea* (50). Intriguingly, we would have expected reduced antagonist activity also by the *rim101Δ* mutant, given its lower ability to colonize apple wounds (Fig. 4). This suggests that besides wound competence, additional factors that influence the antagonistic activity of *P. terrestris* LS28 exist. Moreover, the *in vitro* data obtained for the *rim101Δ* mutant (Fig. 3C) seem to suggest that the alkaline pH-responsive pathway and a functional cell wall do not have a major role in the biocontrol activity of *P. terrestris* LS28.

Understanding the molecular mechanisms of biocontrol agents against fungal pathogens is a crucial requirement to potentiate their antagonistic activity. While there are a number of molecular studies aiming at the elucidation of the molecular basis of biocontrol of filamentous fungi, such as *Trichoderma* (51–53), at the present, the knowledge of the mode of actions of biocontrol yeasts relies on biological investigations that have yet to be supported by molecular analyses (4). Pioneering molecular studies were carried out with the biocontrol yeasts *Candida oleophila* and *Pichia anomala* to demonstrate the involvement of the exo-β-glucanases in their biocontrol activity against *Penicillium digitatum* (54, 55) and *B. cinerea* (21, 56), respectively. Subsequently, for the biocontrol yeast *Pichia angusta* it was found that a leucine-auxotrophic mutant was unable to control brown rot lesion caused by *Monilinia fructicola* compared to its parental WT strain (57), and more recently, for *Metschnikowia pulcherrima* it was demonstrated that a colorless mutant lacking pulcherriminic acid exhibited reduced biocontrol activity against *Botrytis caroliniana* (58). As regards biocontrol yeasts belonging to Basidiomycota, there is only one study that reports the isolation of an uncharacterized mutant of *R. glutinis* that displayed reduced biocontrol activity against *B. cinerea*, and this was correlated with its inability to physically attach to fungal hyphae (59). The results of our study provide molecular evidence on of the role of oxidative stress resistance as an important factor to promote a timely colonization of apple wounds, which are the main sites of penetration of pathogens within the host, with consequent successful competition for nutrients and space.

In conclusion, the availability of molecular tools for both forward and reverse genetics developed in the present study opens the way to a deeper understanding of the genetic mechanisms underlying the biocontrol activity of *P. terrestris* and other basidiomycetous BCAs, with the final goal being its practical use in disease management programs based on biological and integrated control.

**MATERIALS AND METHODS**

**Strains and growth conditions.** The strain used in this study is the basidiomycetous yeast *P. terrestris* strain LS28. This strain was routinely cultured in rich YPD medium (yeast extract at 10 g/liter, peptone at 20 g/liter, dextrose at 20 g/liter, and agar at 20 g/liter for solid medium) at 28°C. To select *P. terrestris* LS28 transformants, solid YPD medium was supplemented with the antifungals hygromycin B (HYG;
In Vitro, neomycin sulfite G418 (NEO; Invitrogen), and nourseothricin (NAT; clonNAT). Antifungals were dissolved in sterile water, filtered sterilized with 0.22-μm filters, and stored at -20°C until their use. The MICs of HYG, NEO, and NAT against P. terrestris LS28 were determined on YPD agar medium supplemented with 100, 200, and 300 μg/ml of HYG and NAT, and 150, 300, and 450 μg/ml of NEO.

Saccharomyces cerevisiae strain FY834 (MATa his3Δ200 ura3-52 leu2Δ1 lys2Δ200 trp1Δ63) (60) was used for in vivo recombination to generate constructs for insertional and targeted mutagenesis in the binary vector pGI3 as described by lanin et al. (61, 62). Strain FY834 was routinely grown at 30°C on YPD medium, while S. cerevisiae transformants were selected on SD-ura (6.7 g/liter of yeast nitrogen base [YNB] with ammonium sulfate and without amino acids, 20 g/liter of dextrose, 20 g/liter of agar).

Agrobacterium tumefaciens strain EHA105 was used to mediate transformation of P. terrestris strain LS28. This strain was cultured onto solid Luria-Bertani medium (LB; 10 g/liter of tryptone, 5 g/liter of yeast extract, 5 g/liter of NaCl, 20 g/liter of agar) at 30°C. A. tumefaciens transformants obtained with pGI3-based vectors were selected on LB supplemented with 50 μg/ml of kanamycin (Sigma-Aldrich).

Development of specific dominant drug markers for P. terrestris LS28. Initially, genetic HYG, NEO, and NAT markers available for C. neoformans were tested for their efficacy in the closely related BCA P. terrestris according to previously reported protocols (27, 29, 39). For the development of specific markers for P. terrestris, the histone H3 protein sequence of S. cerevisiae was retrieved from the Saccharomyces Genome Database (SGD; https://www.yeastgenome.org) and used for a tblastn search against the unpublished P. terrestris LS28 genome assembly. About 1 kb upstream and downstream the identified P. terrestris H3 coding sequence was amplified by PCR and used as the promoter and terminator for the HYG, NEO, and NAT marker genes. Briefly, the P. terrestris H3 promoter and terminator were amplified from P. terrestris LS28 genomic DNA with primers JOHE46128-JOHE46129 and JOHE46132-JOHE46133, respectively, for recombination with the NAT marker, JOHE46128-JOHE46134 and JOHE46137-JOHE46138 for recombination with the HYG marker, and JOHE46128-JOHE46138 and JOHE46141-JOHE46133 for recombination with the NEO marker. The marker genes were amplified from plasmids pPZP-NATcc, pPZP-HYG2, and pPZP-NEO1 (28) with primers JOHE46130-JOHE46131, JOHE46135-JOHE46136, and JOHE46139-JOHE46140, respectively. Primers specific for the promoter and terminator have 20-bp chimeric regions complementary to the plasmid PGI3 and to the gene markers to allow recombination in S. cerevisiae as described below. PCRs were carried out with Phusion high-fidelity DNA polymerase (New England Biolabs) and consisted of 34 cycles of denaturation at 98°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, with an initial denaturation at 98°C for 2 min and a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis with 1% (wt/vol) agarose gels containing ethidium bromide and gel purified.

The H3 promoter and terminator and the gene markers were assembled through in vivo recombination in S. cerevisiae FY834 in the pGI3 plasmid, digested with KpnI and BamHI following a previously reported strategy (61). S. cerevisiae transformants were selected on SD-ura and subjected to colony PCR with primers JOHE43279-JOHE46133 and JOHE43280-JOHE46128 to assess correct recombination at the 5’ and 3’ junctions, respectively. The DNA extracted from S. cerevisiae transformants was used as a template to amplify the recombinant pH3-NAT-TH3, pH3-HYG-TH3, and pH3-NEO-TH3 cassettes for electroporation and biolistics with primers JOHE46368-JOHE46369 or was used to transform A. tumefaciens EHA105 through electroporation. All primers used in this study are listed in Table S1. The plasmids used in the present study are listed in Table S2.

AMT of P. terrestris LS28. Agrobacterium-mediated transformation (AMT) was performed as previously described (63), with minor modifications. A. tumefaciens strain EHA105 transformed with the generated binary vectors was grown at 30°C in shaking cultures in liquid LB supplemented with 50 μg/ml of kanamycin. The culture was centrifuged (3000 rpm, 5 min), diluted in liquid induction medium [IM; MM salts, 40 mM 2-(N-morpholino)-ethanesulfonic acid (MES; pH 5.3), 10 mM glucose, 0.5% (wt/vol) glycerol, 200 μM ascorbylsingyongine (64)] to obtain an optical density at 600 nm (OD600) of 0.2, and grown in liquid IM until the OD600 reached a value of 0.4 to 0.6. P. terrestris LS28 was grown overnight (ON) in YPD liquid at 28°C, diluted to an OD600 of 0.2, and then grown for an additional 3 to 4 h until the OD600 reached a value of 0.4 to 0.6. Cell suspensions of A. tumefaciens and P. terrestris LS28 were mixed in volumes of 200 μl (1:1 and 1:10 in all four possible combinations) and spotted onto agar IM. The use of nitrocellulose and nylon membranes (0.45 μm) was tested. Dual cultures on agar IM were incubated at room temperature for 3 to 7 days without Parafilm. The culture mix was scrapped off the surface, resuspended in 20 ml of sterile water, and centrifuged at 2,500 rpm for 8 min to remove bacterial cells floating in the supernatant. The cellular pellet was then resuspended in an appropriate volume of sterile water (from 300 μl to 1 ml, depending on the amount of cellular pellet) and plated on selective YPD medium containing 200 μg/ml of HYG or 450 μg/ml of NEO. Cefotaxime (CEF; Invitrogen) was added at a concentration of 200 μg/ml to suppress A. tumefaciens growth. Plates were incubated at 30°C until transformants of P. terrestris LS28 appeared. The individual colonies were transferred with a sterile toothpick into petri dishes containing the specific antifungal drug and CEF.

Electroporation of P. terrestris LS28. A ON shaking culture of P. terrestris LS28 grown at 30°C was diluted in liquid YPD medium to an OD600 of 0.2 and grown for an additional 4 to 5 h until the OD600 reached values of 0.6 to 1.0. Cells were centrifuged (3,200 rpm for 5 min at 4°C) and washed twice with 10 ml of ice-cold electroporation buffer (EB; 10 mM Tris-HCl [pH 7.5], 1 mM MgCl2, 270 mM sucrose). Cells were then incubated for 1 h on ice in 10 ml of ice-cold EB with 1 mM dihydrothreitol (DTT). After an additional washing step in 10 ml of ice-cold EB, P. terrestris LS28 cells were resuspended in 250 μl of ice-cold EB and divided into aliquots of 45 μl for each transformation event. About 2 μg of amplified DNA to be transformed was resuspended in 5 μl of water, mixed with the cells in a precooled 2-mm-gap electroporation cuvette, and electroporated with a Bio-Rad Gene Pulser II apparatus using different...
conditions: (i) 0.45 kV, 125-μF capacity, and 600-Ω resistance, as reported for C. neoformans (30); (ii) 0.48 kV, 25-μF capacity, and 600-Ω resistance; and (iii) 0.80 kV, 125-μF capacity, and 1,000-Ω internal resis-
tance, as reported for Phaffia rhodozyma (37). Transformed cells were suspended in 1 ml of YPD medium and
recovered in shaking culture at 30°C for 4 h or ON before plating of the appropriate volume on selective agar medium containing 200 μg/ml of HGY or 450 μg/ml of NEO.

**Biotic transformation of P. terrestris LS28.** The transformation was carried out as described for C. neoformans by Toffalletti et al. (29) using a Bio-Rad PDS-1000 He biologic particle delivery system appar-
atus. Briefly, an ON culture of P. terrestris LS28 was grown at 30°C in liquid YPD medium, centrifuged, washed with sterile distilled water, and resuspended in 1 ml of YPD medium; 350 μl of this cellular sus-
pension was plated onto YPD agar containing 1 M sorbitol to ensure osmotic protection of cells during the transformation event. The plates were then additionally incubated for 3 h at 30°C before biotics. About 2 μg of amplified DNA to be transformed was precipitated on gold microparticles (bioWORLD, Dublin, OH) in the plasmic of CaG3, and free-base spermidine (Sigma) and transformed into P. terrestris L528. Transformation was carried out by testing two different rupture disks (1,330 lb/in² and 1,550 lb/
in²). For recovery, 6 h of incubation at 30°C versus an ON incubation at 24°C was tested; then cells were scraped off the plate with YPD medium plus sorbitol and transferred onto YPD selective agar medium containing 200 μg/ml of HGY or 450 μg/ml of NEO.

**Molecular and phenotypic characterization of P. terrestris LS28 transformants.** Phenotypic sta-
tility tests of P. terrestris LS28 transformants obtained through AMT, electroporation, and biotics were carried out by five passages in nonselective media before being transferred back onto selective media. Transforms able to maintain vigorous growth were considered stable. Representative randomly selected stable transformants were subjected to DNA extraction, and the presence of the exogenous DNA was confirmed by PCR analysis using primers JOHE46135 and JOHE46133, designed on the HGY coding region on the H2 terminator, respectively, and on the H2 terminator region and on the H2 terminator, respectively.

**Development of a system for targeted mutagenesis in P. terrestris LS28.** The unpublished P. ter-
restris LS28 genome was subjected to a tBLASTn search with the S. cerevisiae and C. neoformans Yap1 and Rim101 protein sequences retrieved from SGD and FungiDB. The identified sequences of P. terrestris LS28 were subjected to bidirectional reciprocal BLAST analyses to con-
firm their identification as Yap1 and Rim101 genes. Domain analysis was carried out using InterProScan (https://www.ebi.ac.uk/interpro/search/sequence/).

For mutagenesis, regions of 1,500 bp upstream and downstream the two genes were amplified by PCR on P. terrestris LS28 genomic DNA using primers JOHE46384-JOHE46385 and JOHE46386-JOHE46387 for Yap1, as well as primers JOHE46376-JOHE46377 and JOHE46378-JOHE46379 for Rim101. The HGY cassette was amplified from plasmid pG6 with primers JOHE46368-JOHE46369.

The three PCR fragments were fused using the BamHI-KpnI-digested pG13 plasmid through in vivo recombination in S. cerevisiae as described above. Colony PCR was performed for the identification of S. cerevisiae transformants with primers JOHE43279-JOHE46136 and JOHE46135-JOHE463280 to assess cor-
rect assembly of the recombinant plasmids at the 5’ and 3’ junctions, respectively. Saccharomyces cerevi-
siae transformants were subjected to genomic DNA extraction, and the full gene deletion cassettes yap1::HYG and rim101::HYG were amplified by PCR using primers JOHE43263-JOHE43264, designed on the plasmid backbone. Electroporation and biotic transformation were carried out as reported above.

To identify homologous recombination events, transformants selected on YPD medium plus 200 μg/ml of HGY were single colony purified and subjected to diagnostic PCRs with primers external to the replaced regions in combination with primers specific for the HGY marker, as well as with gene-specific internal prim-
ers. In particular, to identify yap1Δ mutants, primers JOHE46388-JOHE46389 and JOHE46390-JOHE46391 were used to validate the correct junctions at the 5’ and 3’ ends, and JOHE46390-JOHE46391 was used for the internal Yap1 gene. To identify rim101Δ mutants, primers JOHE46380-JOHE46136 and JOHE46381-JOHE46135 were used to validate the correct junctions at the 5’ and 3’ ends (Table S1).

To complement the P. terrestris LS28 yap1Δ and rim101Δ mutants, the Yap1 and Rim101 genes, including native promoters and terminators, were ampliﬁed by PCR on P. terrestris LS28 genomic DNA with primers JOHE46679-JOHE46680 and JOHE46683-JOHE46684, respectively. The NEO marker was ampliﬁed from plasmid pG15 with primers JOHE46681-JOHE46133. PCR ampliﬁcations were performed with Phusion high-ﬁdelity DNA polymerase. The full genes were fused with the NEO marker through in vivo recombination in S. cerevisiae using the pG3-digested plasmid as previously reported. To identify correct plasmids, yeast transformants were screened by colony PCR using primers JOHE43279-
JOHE46680 for the Yap1 gene and JOHE43279-JOHE46684 for the Rim101 gene, both for the 5’ junction, while primers JOHE46141 and JOHE43280 were used to assess junctions at the 3’ end. Genomic DNA was extracted from S. cerevisiae as described before, and constructs for complementation were ampliﬁed using primers JOHE46679-JOHE46133 for Yap1 and JOHE46683-JOHE46133 for Rim101. The two complement-
ary constructs were PCR puriﬁed and transformed through electroporation in the P. terrestris LS28 yap1Δ and rim101Δ mutants. Transformants were selected on YPD medium plus 450 μg/ml of NEO, single-
colony puriﬁed, tested for resistance to both markers HGY and NEO, and then subjected to pheno-
typic characterization.

**Nucleic acid manipulation.** Genomic DNA extraction for PCR analyses was performed as reported by Hoffman (65). Unless otherwise speciﬁed, PCR analyses were performed using Ex Taq DNA polymer-
ase (Takara Bio, Japan) according to the manufacturer’s instructions. PCR conditions were as follows: initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and exten-
sion at 72°C for 1 min/kb. Where high-fidelity Phusion Taq was used, conditions were as follows: initial denaturation at 98°C for 2 min, denaturation at 98°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min/kb.
In vitro and in vivo phenotypic characterization of the \textit{P. terrestris} LS28 \textit{yap1Δ} and \textit{rim101Δ} mutants and respective complemented strains. Two independent \textit{yap1Δ} and \textit{rim101Δ} mutants (one obtained through biolistic transformation and one obtained through electroporation) and one complementation strain \textit{(rim101ΔHYG + rim101Δ-NEO and yap1ΔHYG + yap1Δ-NEO)} were used for \textit{in vitro} and \textit{in vivo} phenotypic characterization using the standard 10-fold serial dilution method. WT \textit{P. terrestris} LS28 was used as the control. Tested conditions and stresses included oxidative stress (hydrogen peroxide \([\text{H}_2\text{O}_2]\), 3 mM; tert-butyl hydroperoxide, 0.5 mM), genotoxic stress (hydroxyurea \([\text{HU}]\), 25, 50, 75, 100, 125, and 150 mM; nocodazole, 0.06, 0.08, 0.10, and 0.15 \(\mu\text{g/ml}\); methyl methanesulfonate \([\text{MMs}]\), 0.2 and 0.5 \(\mu\text{g/ml}\), nitrosative stress (sodium nitrite \([\text{NaNO}_2]\), 0.125 M), endoplasmic reticulum stress (tunicamycin, 0.1, 1, 5, and 20 \(\mu\text{g/ml}\); DTT, 10, 15, and 20 mM), cell wall and plasma membrane stressors (sodium chloride \([\text{NaCl}]\), 1.3 M; lithium chloride \([\text{LiCl}]\), 350 mM; potassium chloride \([\text{KCl}]\), 1.5 M; sorbitol, 2.5 M; Congo red, 0.8%), alkaline stress (pH 8 to 9), and UV stress (150 \(\mu\text{J/cm}^2\)). HYG and NEO plates were used as a control for marker stability. Plates were incubated at 30°C for 3 to 6 days and photographed.

The growth kinetics of the WT and mutant strains of \textit{P. terrestris} were monitored in liquid media YPD, YNB, and apple mimicking. Apple mimicking was prepared as follow: 200-g of Golden Delicious apples was boiled for 30 min and filtered through sterile gauze to remove solid parts, the volume was adjusted to 1 liters, and autoclaving was done at 121°C for 20 min. \textit{P. terrestris} strains at concentration of \(5 \times 10^6\) CFU/ml were inoculated into 10 ml of the aforementioned liquid media, and every day 150 \(\mu\text{l}\) was withdrawn for reading the \(\text{OD}_{600}\) in a microplate reader (Bio-Rad Laboratories, Hercules, CA).

In vivo characterization of the WT and mutant strains of \textit{P. terrestris} LS28 was carried out on Golden Delicious apples purchased from a local market (Campobasso, Italy). The average values of fruit firmness and soluble solid concentration were 3.4 kg and 14° Brix, respectively. Experiments performed included the time course assessment of apple wound colonization by the WT and mutant strains of \textit{P. terrestris} LS28, and bio-control assays against the postharvest pathogens \textit{P. expansum} and \textit{Monilinia fructigena}, carried out as previously described, with minor modifications (12). In these experiments, \textit{P. terrestris} strains were grown ON in liquid YPD medium and washed with sterile distilled water, and the cellular suspensions were adjusted to the desired concentrations (indicated below) in water. Apples were superficially disinfected by immersion for 1 min in a sodium hypochlorite solution (1% [vol/vol] active chlorine), rinsed twice with sterile distilled water, and dried at room temperature. For each strain and for each time point, three biological replicates each consisting of three apples were used \((n=9)\). For time course analysis of apple wound colonization, six wounds (3 mm wide by 3 mm deep) on each fruit were made around the blossom end using a cork borer. Immediately after wounding, 30-\(\mu\text{l}\) volumes of cellular suspensions of \(5 \times 10^6\) CFU/ml of \textit{P. terrestris} strains were inoculated into apple wounds. At 0 min, 30 min, 3 h, 6 h, 24 h, 48 h, and 120 h, apple tissues from the same biological replicate were withdrawn, mixed in 25 ml of sterile water, incubated on an orbital shaker for 30 min at 30°C, and plated \((100\mu\text{l})\) in triplicate onto YPD agar. The plates were incubated at 30°C until colonies to be counted appeared. For each biological replicate and each time point, wound colonization was expressed as mean values of CFU per wound \(\pm\) standard deviations. The means of the CFU/wound values relative to each time point were compared to each other using an ordinary two-way analysis of variance (ANOVA) with Tukey’s multiple-comparison test. The experiment was repeated three times, and a representative experiment is reported. Statistical analysis was performed with GraphPad Prism 8.4.1.

For the biocontrol assays, four wounds (3 mm wide by 3 mm deep) on each fruit were made around the blossom end using a cork borer. The following treatments were alternatively placed in apple wounds: (i) 30 \(\mu\text{l}\) of distilled water containing \(5 \times 10^6\) CFU/ml of \textit{P. terrestris} mutant/complemented strain prepared as described above and (ii) 15 \(\mu\text{l}\) of \textit{P. expansum} or \textit{M. fructigena} suspension of \(2 \times 10^6\) conidia/ml, which was added 30 min after the addition of the \textit{P. terrestris} strains. \textit{P. expansum} and \textit{M. fructigena} conidial suspensions were prepared by collecting conidia with sterile water plus 10% Triton X-100 from a potato dextrose agar (PDA) plate, washing them with sterile water, and adjusting them to the desired concentration using a hemocytometer. Controls were wounds treated only with 30 \(\mu\text{l}\) of yeast cellus suspensions, 15 \(\mu\text{l}\) of conidial suspensions, or 30 \(\mu\text{l}\) of sterile distilled water. Fruits were incubated at room temperature \((22\text{ to }25°C\)) with 95 to 98% relative humidity. The number of wounds showing rot symptoms and the diameters of lesions were monitored daily and recorded on the 7th day of incubation for \textit{P. expansum} and the 10th day of incubation for \textit{M. fructigena}. The assays were stopped when control fruits reached 90% to 100% infected wounds.

Biocontrol experiments were performed three times, and a representative experiment is reported. Each experiment included three biological replicates each consisting of three technical replicates (i.e., three apples for each strain \((n=9)\)). For \textit{P. expansum}, disease severity was calculated using the lesion diameters: for each strain, the means of the diameters recorded for each replicate \((n=12\text{; 3 apples with 4 wounds each})\) were compared to each other using an ordinary one-way ANOVA with Tukey’s multiple-comparison test. For both \textit{P. expansum} and \textit{M. fructigena}, biocontrol activity was expressed as the percentage of infected wounds on the total. For each strain tested, the means of each biological replicate were compared to each other using an ordinary one-way ANOVA with the Tukey’s multiple-comparison test. Statistical analysis was performed with GraphPad Prism 8.4.1.

**Data availability.** The \textit{P. terrestris} transcription factors \textit{YAP1} and \textit{RIM101} have been deposited in Gen\textsuperscript{B}ank under accession numbers MW298104 and MW298103, respectively. All microbial strains and mutants used in the present study are available under request.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.**

**SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.**
REFERENCES